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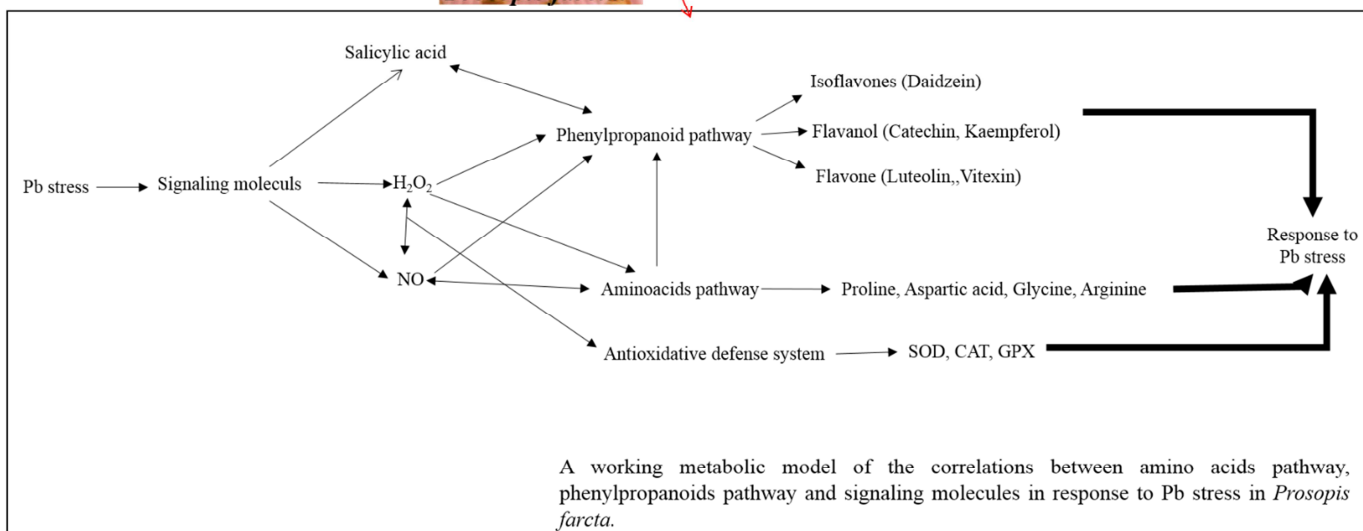
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Modulation of Pb-induced stress in *Prosopis* shoots through an interconnected network of signaling molecules, phenolic compounds and amino acids

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ABSTRACT

Lead (Pb) is a hazardous heavy metal present in the environment which elicits oxidative stress in plants. To characterize the physiological and biochemical basis of Pb tolerance, *Prosopis farcta* seedlings were exposed to Hoagland's solutions at six different Pb concentrations (0, 80, 160, 320, 400 and 480 μ M) for different periods of time. As expected, application of Pb significantly increased hydrogen peroxide (H_2O_2) content. In response, *P. farcta* deployed the antioxidative defence mechanisms with significantly higher activities of superoxide dismutase (SOD), enzymes related to H_2O_2 removal, and also the increases in proline as a solute marker of stress. Increases were observed in nitric oxide (NO) production which could also act in triggering defense functions to detoxify Pb. Enhanced phenylalanine ammonia-lyase (PAL) activity at early days of exposure to Pb was correlated with increases in phenolic compounds. Significant increases in phenolic acids and flavonoids; daidzein, vitexin, ferulic acid and salicylic acid were observed with Pb treatment. Furthermore, the stress effects were followed by changes in free amino acid content and composition. Aspartic acid and glycine content was increased but glutamic acid significantly decreased. It is likely that stress signal transduction by NO and H_2O_2 mediated defence responses to Pb by coordination of antioxidative system and metabolic pathways of phenylpropanoid and amino acids.

Keywords: Lead, enzymatic antioxidant, nitric oxide, phenylalanine ammonia-lyase, phenolic acids, amino acids

Abbreviation: H_2O_2 : hydrogen peroxide, ROS: reactive oxygen species, NO: nitric oxide, PAL: phenylalanine ammonia-lyase, SOD: superoxide dismutase, CAT: catalase, GPX: guaiacol peroxidase, EtOAc: ethyl acetate, MeOH: methanol.

1. Introduction

The impact of heavy metal pollution on human health and also its exorbitant persistence in the environment is a major ecological concern (Gupta et al., 2009). Lead, one of the most abundant globally distributed toxic elements, has attracted considerable interest by researchers. Accumulation of Pb causes a number of physiological, biochemical and structural disorders like unfavourable influences on the photosynthetic processes, chlorophyll contents, uptake of essential elements, biomass and root elongation (Ali et al., 2014a; Arias et al., 2010). One of the phytotoxic effects of Pb appears to be the disruption of balanced cellular redox status with concomitant induction of oxidative stress (Verma and Dubey, 2003). In line with this Pb and other heavy metal stresses have been reported stimulate reactive oxygen species (ROS) accumulation in variety of plants (Ali et al., 2014a, b). It is well-established that H_2O_2 accumulation, as the most stable form of ROS, can have a negative effect on plant physiology but; particularly at low levels, can act as messengers involved in signal transduction pathways (Kováčik et al., 2009). To ensure survival and growth under adverse environmental conditions, plants have developed protective mechanisms enabling them to counteract negative effects caused by metals abundance in their tissue. In addition, it has recently been suggested that nitric oxide (NO) - another bioactive molecule involved in signaling within plants - plays a center role in a variety of physiological and biochemical functions including protection against oxidative damage induced by stress (Singh et al., 2008; Singh et al., 2013). To control the level of ROS and to protect the cells under stress condition, NO activates antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Singh et al., 2013). SOD is the major superoxide radical scavenger and its enzymatic action results in H_2O_2 formation, which is toxic and must be eliminated by conversion to H_2O in subsequent reactions by CAT and GPX.

Plant phenolics are considered as parts of active defense responses and to have roles in H_2O_2 reduction in the phenol-coupled ascorbate peroxidase reaction (Dučić et al., 2008). Pb was reported to induce mRNA coding for phenylalanine ammonia-lyase (PAL), which is regarded as the rate limiting enzyme leading production of the phenolic group known as phenylpropanoid in the response of legume plants to metals (Pawlak-Spradaetal., 2011). Moreover, another phenolic, salicylic acid (SA) has been linked to the alleviation of heavy metal- induced growth inhibition in two melon genotypes by promoting antioxidant defence capacity, photosynthesis process and proline metabolism (Zhang et al., 2015). It should be noted that many studies also reported that NO together with H_2O_2 trigger signal transduction pathways to stimulate PAL and the accumulation of secondary metabolites in plants under stress conditions. However, metabolic networks which confer plant tolerance to heavy metal stress requires further work to be understood (Kováčik et al., 2009). Plant metabolism is known to undergo considerable reprogramming in

response to metal treatment. Several workers have emphasized the importance of the synthesis of metal chelating compounds to avoid heavy metal toxicity (Sharma and Dubey, 2005). These, chelating compounds include some charged amino acids for example, proline, and are likely to contribute to amelioration of negative impacts owing to heavy metal excess (Manara, 2012; Kováčik et al., 2010). Such chelating solutes contribute as an available source of carbon and nitrogen and also play other roles in heavy metal stress responses, including acting as signaling molecules, regulating ion transport and facilitating detoxification during stress (Dubay and Pessarakis, 1995; Xu et al., 2012).

Prosopis species are perennial trees/shrubs that are found abundantly in arid and semi-arid zones of the world. They are well known for their resistance to heavy metal and have been used as indicator species to assess ecotoxicity of soils polluted by contaminants (Beramendi-Orosco et al., 2013; Usha et al., 2009). This stated there is little information available on strategies employed by *Prosopis*; particularly in *P. farcta*, that allows them to cope with Pb toxicity. The present work was conducted to evaluate the influence of Pb stress on the antioxidant defence system and the endogenous signaling molecules H_2O_2 and NO. Moreover, the effect of Pb stress on the primary and secondary metabolism (amino acids and the phenylpropanoid pathway) and the activity PAL associated with phenolic compounds biosynthesis was investigated. By comprehensively, comparing these metabolites, we have improved our understanding of coordinated pathways involve in detoxification of heavy metal stress. Hydroponic system was used to provide potential to examine metal tolerance and magnitude of metal accumulation in plant species with greater precision than soil systems.

2. Materials and methods

2.1. Chemicals

The Fluka A2161 amino acid reference solution for fluorescence detection, o-phthalaldehyde (OPA), salicylic acid, ferulic acid, cinnamic acid, caffeic acid, coumaric acid, gallic acid, daidzein, vitexin, myricetin, resveratrol, quercetin, kaempferol, naringinine, catechin, luteolin, diosmin, apigenine, rutin, orientin, genistin was all purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Plant materials and growth conditions

Seeds of *Prosopis farcta* were collected in western Ilam province, Iran. The healthy seeds of uniform size were selected and scarified with 98% sulphuric acid for 13 min and surface sterilized with 2% of sodium hypochlorite solution, followed by repeated washings with distilled water. Seeds were germinated by placing in a Petri dish with two layers of water-saturated filter paper. Germinated seedlings with 20 mm-

long roots were then transferred into plastic containers with 2.5 dm³ of Hoagland nutrient solution (pH 6.8). The seedlings were left to grow in a growth chamber under a cycle of 16 h light (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with a 27/22°C day/night temperature and 60–80% air humidity. The nutrient solution was renewed every 5 days to prevent nutrient depletion. Each experiment was performed three times, consecutively (3 containers per treatment each time).

2.3. Treatment pattern and experimental design

This study was conducted in two separate experiments. In the first, the 21-day-old seedlings were supplemented with 0, 80, 160, 320, 400 and 480 μM of lead (Pb in the form of $\text{Pb}(\text{C}_2\text{H}_3\text{CO}_2)_2$) with nutrient solution for 4 days (96 h). Based on the derived data step, one concentration of Pb (400 μM) was chosen to measure antioxidant enzymes and proline content. In the second approach, 21-day-old seedlings were grown in the same nutrient solution supplemented with chosen concentration of lead, but harvested after 0 (i.e. prior to start of treatment), 12, 24, 48, 72, 96 h of treatment and washed three times with distilled water. At each time point, shoots of plants were collected at random from each tray, frozen with liquid N_2 , and stored at -80°C for analysis of amino acids, phenolic acids and flavonoids, NO, H_2O_2 content and PAL activity.

2.4. Analysis of Pb content

Frozen primary leaves were ground to a fine powder in a mortar pre-cooled with liquid N_2 , weighed, and transferred to porcelain crucibles where they were dried out at 100°C until constant weight was attained. Subsequently, dried samples were burnt to ashes at 500°C for 6 h. The ashes were then dissolved with 0.1 M HCl. Pb was analyzed in this acid extract according to the method described by Camacho- Cristobal and Gonzalez-Fontes (2002). Pb was measured using an atomic absorption spectrometer (Shimadzu AA-6709).

2.5. Antioxidant enzyme assay

Liquid N_2 frozen shoots (0.2 g) were crushed into a fine powder in a mortar and pestle. Soluble proteins were extracted by 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12000 \times g for 20 min at 4°C and then the supernatant was used for the following enzyme assays.

Total SOD (E.C.1.15.1.1.) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). One unit of SOD

was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT (E.C.1.11.1.6.) activity was assayed according to the method of Cakmak and Marschner (1992). The reaction mixture was 25 mM sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2 . The reaction was initiated by the addition of 100 μL of the enzyme extract, and activity was determined by measuring the initial rate of disappearance of H_2O_2 at 240 nm for 1 min ($E = 39.4/(\text{mM cm})$).

GPX (E.C.1.11.1.7) activity was based on the determination of guaiacol oxidation (coefficient of absorbance $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) at 470 nm by H_2O_2 . The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol and 0.1 mL of 10% H_2O_2 in a 3 mL volume. The reaction was initiated by adding 100 μL enzyme extract and was followed for 3 min (Lin and Wang, 2002). Total soluble protein contents were determined according to the method of Bradford (1976), using bovine serum albumin to provide standards.

2.6. Determination of proline concentration

Proline was extracted and determined by the method of Bates et al. (1973). Plant samples were homogenized with 3% sulphosalicylic acid and the homogenate was centrifuged at $3000\times g$ for 10 min. After acetic acid and acid ninhydrin were added, the supernatant was boiled for 1 h and then absorbance of the supernatant at 520 nm was determined. Proline concentration was calculated using a proline standard curve and expressed as mg g^{-1} dried weight.

2.7. Nitric oxide content

NO generation also quantified by determination of nitrite (NO_2^-) concentration *in vivo* using Griess reagent. Samples (0.2 mL) were incubated with 1.8 mL of 100 mM PO buffer (pH 7.0) and 0.2 mL of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid solution) at room temperature for 10 min (Green et al., 1982). Absorbance of the reaction mixture was read at 540 nm and concentration of NO determined from a calibration curve prepared using sodium nitrite as standard.

2.8. Hydrogen peroxide (H_2O_2) determination

To determine H_2O_2 concentration, root tissue (100 mg) was extracted with 5 mL trichloroacetic acid (TCA; 0.1%, w/v) in an ice bath and centrifuged at $12000\times g$ for 15 min (Velikova et al., 2000). An aliquot (0.5 mL) of supernatant was added to 0.5 mL of phosphate buffer (pH 7.0) and 1 mL of 1 M KI.

The absorbance of the mixture was read at 390 nm. H_2O_2 content was determined using the extinction coefficient $0.28 \text{ M}^{-1} \text{ cm}^{-1}$ and amount expressed as $\text{nmol g}^{-1} \text{ FW}$.

2.9. Quantitative determination of total phenolics

Folin-Ciocalteu reagent was used to measure total phenolic content according to the method of Akkol et al. (2008). One mL of methanolic extract was mixed with 5 mL Folin-Ciocalteu reagent and 4 mL sodium carbonate solution 7.0%. The mixtures were allowed to stand for 2 h and then the absorbance was measured at 765 nm. Gallic acid was used as a standard for the calibration curve. Total phenol values are expressed in terms of mg gallic acid equivalents in 1 g DW.

2.9.1. Qualitative extraction of phenolic acid and flavonoids using HPLC

Plant samples (0.2 g FW) were extracted with methanol (3 x). The methanol extracts were pooled and the solvent was evaporated under vacuum at 35°C . The residue was suspended in acetonitrile (50 mL), and extracted three times with hexane (20 mL) to remove lipid components. The hexane extracts were discarded, and the acetonitrile solution was dried over anhydrous magnesium sulfate. The acetonitrile was removed in vacuum at 35°C (Owen et al., 2003). The dried residue was suspended in methanol (5 mL) to separation of phenolic acids by HPLC (Agilent Technologies 1260 infinity, USA). The stationary phase was a C18 column (Perfectsil Target ODS-3 (5 μm), $250 \times 4.6 \text{ mm}$) MZ Analysentechnik, Mainz, Germany). The elution solvent was 2% acetic acid in water (solvent A) and methanol (solvent B) (Owen et al., 2003) with a gradient system as followed: 0–2 min 5% B, 2–10 min 25% B, 10–20 min 40% B, 20–30 min 50% B, 30–40 min 100% B, 40–50.0 min 5% B. Phenolic acids in the eluant were detected with a UV dual-array detector (HP 1040M) set at 278, 300 and 340 nm at a flow rate of 1 mL/minute.

Extraction of flavonoid compounds was performed following Keinanen et al. (2001). Briefly, green tissue (0.2 g DW) was ground in liquid N_2 and transferred to a centrifuge tube with 1.5 mL of 40% aqueous methanol containing 0.5% acetic acid. After shaking for 3 h, samples were centrifuged (12 min, $4000\times g$) and the supernatant was used for HPLC analysis. The separation of flavonoids compounds was carried out using the method of Gudej and Tomczyk (2004). The gradient mobile phase contained 0.5% phosphoric acid in water (A solvent) and acetonitrile (solvent B), and the UV detector was set at 254, 280, 300 and 350 nm. The elution was as follows: 0–30 min 1 8% B, 30–60 min 67% B, 60–65 min 18% B, 65–70 min 18% B. Chromatography was performed at 25°C at a flow rate of 0.8 mL/minute. The amounts of phenolic compounds in the extracts were calculated from standard curves of authentic standards.

2.10. Extraction and assay of PAL

The protein extract was used as a crude enzyme solution. The reaction mixture was composed of 0.15 mL of crude enzyme and 1 mL of extraction buffer. The reaction started with the addition of 0.35 mL of 100 mM phenylalanine and after 1 h incubation at 37°C, was stopped with the addition of 100 µL of 5 M HCl. The mixture was extracted three times with ethyl acetate (EtOAc). The EtOAc extract was air-dried, re-dissolved in pure MeOH and analyzed using HPLC as described for the determination of the phenolic acids (Wakabayashi et al., 1997). The enzyme activity was expressed in terms of the amount of cinnamic acid (CA) produced for 1 h per mg of protein.

2.11. Quantitative estimation of total amino acids

Free amino acids content was estimated according to the method of Lee and Takahashi (1966). Briefly, 0.1 g plant material was incubated overnight in 70% ethanol followed by washing with distilled water. Volumes of 0.5 mL acetate buffer, 0.5 mL ninhydrin solution and 3 mL of 55% glycerol-water diluents were added to 1 mL of the extract. This was then heated in a water bath at 100°C for 30 minutes. Immediately after removal from the water bath, test tubes were cooled in running tap water and gently shaken. The absorbance was read using a spectrophotometer at 570 nm. Glycine in 0.5 M, pH 5.6 citrate buffer was used as the standard.

2.11.1. Chromatographic separation of amino acids

Plant samples (0.2 g FW) were ground in liquid N₂ to a fine powder and mixed with 2 mL of ethanol and water (80:20 v/v), left for 10 min, collected and centrifuged. The extraction procedure was repeated on the pellet. Aliquots of the extracts were evaporated to dryness under vacuum and the residue was dissolved in 1 mL H₂O (Di Martino et al., 2003). The measurement of amino acid concentrations was performed using an HP 1100 liquid chromatograph with fluorimetric detector FLD HP 1100 and using precolumn derivatization with OPA. Separation was carried out with a Zorbax Exlipse AAA column (4.6 × 150 mm, 3.5-µm particle size; Agilent Technologies, USA). Mobile phase A was aqueous buffer (25 mM Na₂HPO₄/ NaH₂PO₄, pH 7.2)/ tetrahydrofuran (95:5, v/v) and mobile phase B was aqueous buffer (25 mM Na₂HPO₄/ NaH₂PO₄, pH 7.2)/ methanol/ acetonitrile (50:35:15, v/v/v). The elution was facilitated by gradient program as follows: 0–0.6 min 10% B, 0.6–9.0 min 50% B, 9.0–48 min 60% B, 48.0–51.0 min 100% B, 51.0–56.0 min 100% B, 56.0–57.0 min 10% B, 57.0–59.9 min 10% B. The constant flow rate was 0.5 mL/min. Fluorescence detection and quantification was carried out by excitation wavelength 230 nm and emission wavelength 455 nm. Sample peaks were identified by comparison of retention time with reference substances (Biermann et al., 2013).

2.12. Statistical analysis

All analyses were conducted at least three times, each with three independent repetitions. The analysis of variance and the Duncan test ($P \leq 0.05$) of mean comparison were performed using the MSTATC program ver. 1.4.

3. Results

3.1. Pb content

In initial experiments, 21-day-old seedlings of *P. farcta* were exposed to different concentrations of Pb (0, 80, 160, 320, 400 and 480 μM) for 96 h. The seedlings accumulated increasing concentrations of Pb, in a dose-dependent manner. The Pb levels in shoots showed positive linear relationships with the Pb concentration in the nutrient solution up to 400 μM (Table 1).

3.2. Antioxidant enzyme activities and proline content

Table 1 shows the effect of lead stress on three different antioxidant enzymes such as SOD, CAT and GPX in *Prosopis* shoots. Results show that the activity of SOD, a key enzyme for catalyzing the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), significantly increased under different concentration of Pb. The activity of CAT followed a similar trend to SOD and their greatest activities were observed at 400 μM Pb. The pattern of increased GPX activities proved to be different with increased activity first seen at 160 μM Pb and was still increased with at 480 μM Pb. With proline content, as a compatible solute, significant increases over controls were first with at 320 μM Pb (72%) exposure and did not significantly differed at high concentrations of Pb. Based on these preliminary observations, in order to monitor biochemical and metabolites changes in *Prosopis* in response to Pb, a concentration of 400 μM Pb was selected. Furthermore, the effects of 400 μM Pb were examined over a 0, 12, 24, 48, 72, 96 timecourse.

3.3. NO and H_2O_2 generation.

To begin to assess if NO and H_2O_2 have roles in response to Pb stress, the levels of these signals was measured in seedlings following Pb treatment. The data in Table 2 clearly show NO production increases over time following Pb treatment. The amount of detected NO peaked at 12 h (a 28% increase over controls) but then decreased at 96 h following Pb treatment (14% over controls). In parallel to changes in NO content, the amount of H_2O_2 also increased significantly in response to Pb toxicity, suggesting ROS

signaling events and / or oxidative damage (Table 2). At 12 h of treatment, it reached a 1.62 fold increase compared to that in the control (12 h), however, this was reduced at 72 h to 1.24 fold.

3.4. PAL activity

Based on the observation that heavy metal induced NO and H₂O₂ accumulation, it was hypothesized that Pb might trigger NO and H₂O₂ generation to induce PAL activity to feed into pathways leading to the production of antioxidant metabolites such as phenolics compounds. Therefore, the effect of Pb on PAL activity was also determined. As shown in Table 2, at 24 and 48 h of treatment, PAL activity increased by 63% and 35% over controls and then declined at 96 h compared to equivalent controls.

3.5. The phenolic composition and concentration

The involvement of the secondary metabolism in Pb resistance was tested by measuring the levels of phenolic compounds. Total phenolics content at 12 h of treatment remained unaffected compared to controls but increased thereafter (Table 2). Total phenolic content appeared to particularly increase at 72 and 96 h following treatment. However, phenolic compounds increased by ~25% over controls between 24 and 48 h of treatment but thereafter there appeared to be no significant difference between the time points.

The HPLC analysis was then performed to identify and quantify phenolic metabolites in shoots under stress. In particular, the levels of 19 phenolic standards, 4 phenolic acids (salicylic, ferulic, cinnamic and caffeic acids) and 9 flavonoids derivatives (daidzein, vitexin, resveratrol, myricetin, quercetin, kaempferol, naringin, luteolin, diosmin) were measured. Figure 1a shows a prominently increase in salicylic acid (1.86 fold) at 72 and 96 h of treatment over their respective controls. A significant increase for ferulic and cinnamic acid occurred between 12 and 72 h of treatment (Fig. 1b, c); whereas, caffeic acid content decreased exception at 72 h of treatment compared to controls (Fig. 1d). A strikingly strong increase was observed for daidzein at 96 h of treatment, and the increase was 2.34 fold higher than that in 96 h control (Fig.1e). Likewise, vitexin showed a linear increment in content during the treatment period (Fig. 1f). A significant increase was observed for resveratrol and myricetin at 48, 72 and 96 h of treatment, while their levels were kept at the same level as in the respective controls at 12 and 24 h of treatment (Fig 1g, h). Quercetin Levels showed no change and kaempferol was clearly reduced in the shoots only at 48 h of treatment compared with control (Fig 1i, j). The most striking reduction was seen in naringin content, some four fold at 48 h of treatment, before a ~60 % of increase at 96 h over the respective controls (Fig 1k). The level of luteolin and diosmin showed fluctuating patterns during time-course in both group: treated plants and controls (Fig 1l, m).

3.6. The quantitative and qualitative contents of free amino acids

The data regarding the effect of lead stress on total amino acid contents have been shown in Table 2. The results showed that compared to controls, Pb-stressed plantlets had significantly higher concentrations of total amino acids at all time periods with the exception of 12 h data set. Pb stress induced a marked accumulation of amino acids in the shoots by 52 and 54% at 48 and 72 h of stress, respectively.

The HPLC analysis of *Prosopis* exposed to Pb stress was performed to identification of the specific changes in amino acid metabolites. In shoots, 14 basic amino acids were detected. Pb treatment greatly altered the composition and concentration of these free amino acids (Fig. 2). In general, the greatest quantitative and qualitative alterations in the levels of free amino acids were seen at 48 and 72 h of Pb treatment compared to their untreated controls. Considering changes in individual amino acids in response to Pb stress, the greatest increase was observed for Asp between 12 to 72 h of Pb exposure and was in the range of 2.94 to 14 times over their respective controls (Fig. 2a). Furthermore, considerable increases were observed for Gly and Thr at 24, 72 and 96 h of treatment compared to controls (Fig. 2b); as did Arg, although to a lesser extent (Fig. 2c). In contrast, a significant decrease in Glu level (from 12 to 72 h of treatment) revealed a marked shift between these two acidic amino acids. However, Glu content was two times higher compared to the equivalent control at 96 h of treatment (Fig. 2d). A group of amino acids, including Leu, Ile, Val, Met, Ala, exhibited a clear increase and then decrease at 72 and 96 h of treatment, respectively, but did not significantly change during the first 48 h of treatment (Fig. 2e, f, g, h, i). Pb stress did not result in a marked shift in the level of Ser and His (Fig 2j, k). Interestingly, amino acids deriving from the shikimate pathway; tyrosine and phenylalanine, exhibited the greatest increase at an earlier timepoints; 24 and 48 h of treatment compared to controls (Fig. 2l, m). These data were indicative of differential amino acid metabolism at differing times following the application of Pb.

4. Discussion

Plants generally experience oxidative damage when exposed to lead and other metals (Ali et al., 2014a, b; Bharwana et al., 2013). In the present study, increased ROS was found under metal stress as indicated by H_2O_2 contents, which either causing widespread damage or serving as signaling molecules (Dat et al., 2000). This increase in H_2O_2 accumulation during treatment changes the redox status of the cell and induces the production of antioxidants and the activation of antioxidant mechanisms (Manara, 2012). In addition to H_2O_2 , NO may also be involved in the signal transduction pathway triggered by heavy metals (Neill et al. 2003). Under biotic and abiotic stresses, NO generation and a parallel accumulation of ROS can activate resistance mechanisms in plants either independently or synergistically (Zhang et al., 2007). In this study, accumulation of NO and H_2O_2 at 12 and 24 h of treatment, respectively, may

trigger some common defense machineries in the plants such as stress related proteins in the defensive antioxidant system. Plants are equipped with complex antioxidant system to overcome ROS caused damages which are effective at different levels of stress-induced deterioration (Hegedüs et al., 2001). In the present investigation, the observed enhancement in activities of antioxidant enzyme proteins such as SOD, CAT and GPX in response to Pb exposure is in agreement with other published reports of heavy metals (Singh et al., 2008). Increased activity of CAT and SOD points towards their induction to quench and remove higher levels of O_2^- and H_2O_2 generated due to Pb stress (Sharma and Dubey, 2005; Ali et al., 2014). Our results indicate that CAT increased faster than GPX production, showing that CAT might be more responsible for H_2O_2 elimination during the early hours of Pb stress than GPX. Interestingly, this period of increased GPX activity supports a link between GPX activity and amino acid accumulation, established substrates for its function, in response to Pb stress. Increased antioxidant enzyme activities could arise from *de novo* gene expression or altered levels of enzyme inhibitors in order to achieve a balance between oxidant and antioxidant levels under Pb phytotoxicity.

The observed significant increase in PAL activity at early 48 h of Pb treatment was consistent with the similar findings in *Panax ginseng* roots in response to metal stress (Babar Ali et al., 2006). Further a link with NO was suggested from the work of Delledonne et al., (1998) who showed that inhibition of NO production markedly reduces the accumulation of PAL and chalcone synthase, the first enzyme of the branch specific for flavonoids and isoflavonoids in plants. Complementary to this, Kováčik et al., (2009) found that expression levels of PAL and also its activity can be elicited by both NO and ROS. Our collective findings suggest a crucial role for NO and H_2O_2 in regulating the activity of PAL and consequently the induction of phenylpropanoid biosynthesis pathway during stress. These would represent key events in conferring tolerance to Pb exposure in *P. farcta*.

Following increased PAL activity, our study also considered the role of phenolics in conferring tolerance to Pb. In accordance with our results, there are some reports showing the influence of heavy metal stress on the phenolic metabolism. Phenolic compounds can protect against metal toxicity by metal chelation and direct scavenging of reactive oxygen species. Phenolics, especially flavonoids and phenylpropanoids, are oxidized by peroxidase, reduced by ascorbate, and act in H_2O_2 scavenging system (Michalak, 2006). Accumulation of essential amino acids and phenolic compounds like salicylic acid (which directly involved in stress signaling), catechol and catechin play key roles in responses of plants to biotic and abiotic stress (Poschenrieder et al., 2006; Heim et al., 2002) and there is a good link with responses to heavy metal excess (Kováčik et al., 2010). The cinnamic acid derivatives, epicatechin, and rutin increased in the presence of cadmium and played an important role in the metabolism of *Erica*

andevalensis to survive in heavy metal polluted soils (Márquez-García et al., 2012). The aromatic amino acids phenylalanine, tyrosine and tryptophan were utilized not only for protein synthesis, but also served as precursors for a wide range of secondary metabolites such as flavonoids, phenolic acids, phytoalexins (Babar Ali et al., 2006). An increase in the amount of aromatic amino acids, some flavonoids and phenolic acids in the *Prosopis* shoots in response to Pb, confirms the close relationships between these compounds in responding to this stress.

Plants have evolved a variety of protective mechanisms to ensure survival and growth under adverse environmental conditions. The synthesis and accumulation of amino acids, known as compatible solutes, represent ubiquitous mechanisms for stress amelioration in plants (Di Martino et al., 2003). They may play a more active role in the stabilization of enzymes and/or membranes, in addition to functioning as carbon and energy storage during limited growth and photosynthesis (Gilbert et al., 1998). Thus, the accumulation of excess total amino acid in response to Pb can be regarded as an important adaptive response of plants to avoid Pb toxicity (Sharma and Dubey, 2005), which was consistent with the increase amino acids content in *Prosopis* seen in our experiments. The amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses such as heavy metal stress (Chen et al., 2002). It is possible that proline chelates metal ions to improve heavy metal stress tolerance. Additionally, there is increasing evidence of cross-talk between these of NO and ROS signalling pathways and proline metabolism in plant cells. The considerable accumulation of proline in response to NO and ROS has been observed in plants under adverse environmental conditions like metal stress (Rejeb et al., 2014; Hasanuzzaman and Fujita, 2013). In addition, proline metabolism appears to play a key role in triggering signal molecules, which are involved in allowing the adaptation of plants to various environmental constraints (Rejeb et al., 2014).

The branched-chain amino acid aspartate (Asp) feeds into the synthesis of Asn, Lys, Met, Thr, and Ile as well as the conversion of Thr into Gly (Angelovici et al., 2009). A Zn-asparagine complex may reduce Zn toxicity and asparagine by acting as ligand towards Cd, Pb, and Zn (Sharma and Dietz, 2006). Should this mechanism be correct the increased amino acids that we have observed in our own experiments, would reduce the ability of Pb to contribute to cellular toxicity. The reduction in glutamate content with Pb (Fig. 2d) would be consistent with its utilization as substrate in the synthesis of Glu, Gly, proline and possibly the recycling of ammonia produced during the synthesis of other amino acids (Di Martino et al., 2003). We can suggest that the increase of Gly is associated with GSH and phytochelatin biosynthesis as the most important markers in heavy metal stress. Also, the fluctuating content of amino acid Ser could be linked to Gly and Cys biosynthesis during time course of stress (Ahsan et al., 2012). Also, Met is a central metabolite in antioxidant defense and metal sequestration (Satoh et al., 2007). Thus, taking all these data

together nitrogen metabolism and particularly amino acid anabolism is central to the response of plants to heavy metals.

5. Conclusion

The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metal by signaling molecules such as NO and H₂O₂. By using *P. farcta*, as a resistant plant to heavy metal, we present evidence suggesting that H₂O₂ and NO cooperatively trigger defense functions such as activation of antioxidant system, induction of PAL and enhancement the content of phenolic compounds. Also, changes in NO and ROS production and changes in amino acid metabolism may be concurrent events in plants under Pb stress. Further research by using NO and ROS generators/scavengers, might reveal novel and interesting links, which may contribute to a better understanding the connection of these metabolisms with signal molecules in the adaptation of plants to environmental stresses.

Acknowledgements

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Tables

Table 1. Absorbed Pb, antioxidant enzymes, proline contents of *Prosopis* shoots under different concentration of Pb. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

Table 2. The content of NO, H₂O₂, Total phenolics, Total amino acids and PAL activity of *Prosopis* shoots under control and Pb treated conditions. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

Figures

Fig. 1. Content of phenolic acids and flavonoids in *Prosopis* shoots: (a) salicylic acid, (b) ferulic acid, (c) cinnamic acid, (d) caffeic acid, (e) daidzein, (f) vitexin, (g) resveratrol, (h) myricetin, (i) quercetin, (j) kaempferol, (k) naringin, (l) luteolin, (m) diosmin. The plants were exposed to Pb treatment for 96 h. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

Fig. 2. Accumulation of free amino acids (nmol g FW, n =3) in *Prosopis* shoots: (a) aspartic acid, (b) glycine+threonine, (c) arginine, (d) glutamic acid, (e) leucine, (f) isoleucine, (g) valine, (h) methionine, (i) alanine, (j) serine, (k) histidine, (l) tyrosine, (m) phenylalanine. The plants were exposed to Pb treatment for 96 h. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

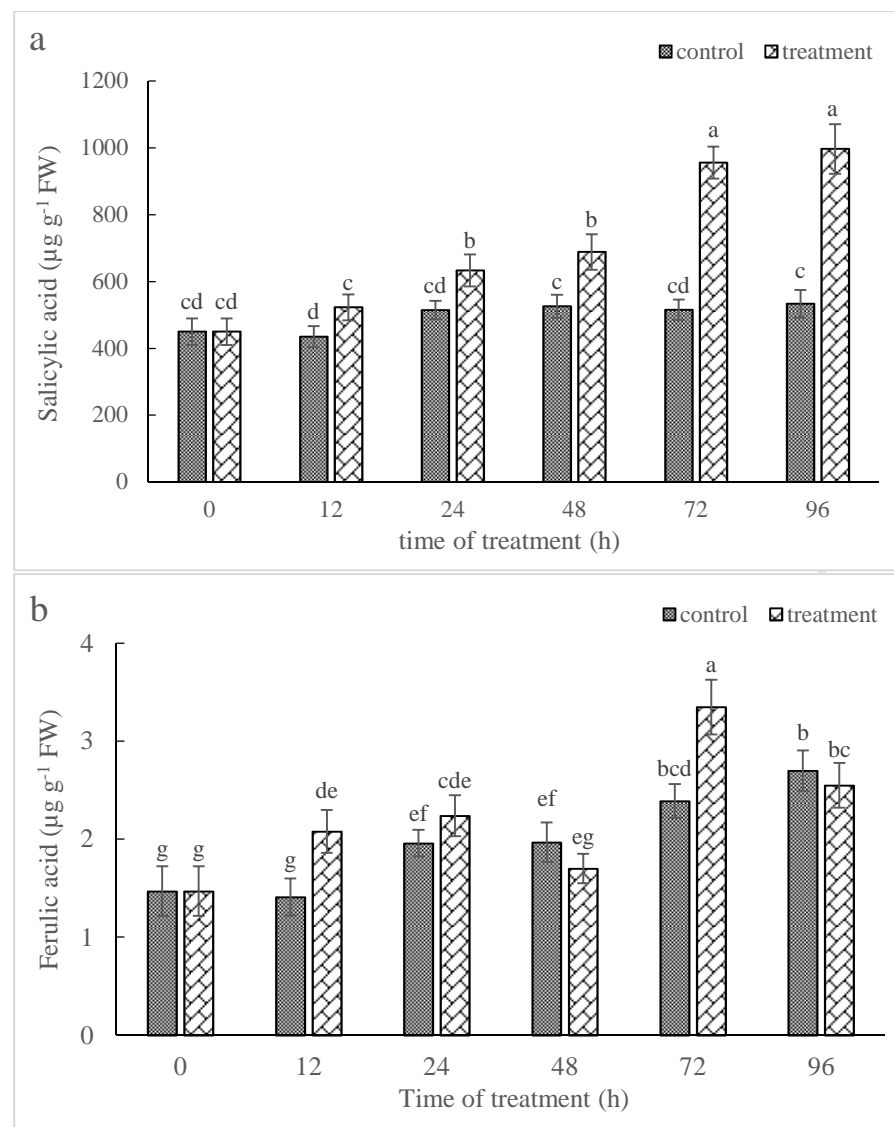
Treatment Pb (μM)	Absorbed Pb (mg/kg)	SOD activity [U $\text{mg}^{-1}(\text{protein})$]	CAT activity [μmol (H_2O_2) mg^{-1} (protein) min^{-1}]	GPX activity [μmol (guaiacol) mg^{-1} (protein) min^{-1}]	Proline content (mg g^{-1} DW)
0	1.3 ± 0.61^e	12.3 ± 0.30^d	2.19 ± 0.30^d	9.6 ± 1.27^d	1.52 ± 0.13^{bc}
80	27.4 ± 1.7^d	21.1 ± 2.60^c	3.81 ± 0.46^{bc}	11.8 ± 1.45^{cd}	1.60 ± 0.19^{bc}
160	38.3 ± 2.5^c	26.7 ± 1.92^b	4.23 ± 0.55^{bc}	13.7 ± 1.82^c	1.97 ± 0.23^b
320	55.4 ± 5.1^b	35.0 ± 2.41^a	5.47 ± 0.71^a	17.1 ± 0.98^b	2.63 ± 0.25^a
400	68.7 ± 4.9^a	36.5 ± 3.01^a	4.72 ± 0.52^{ab}	19.9 ± 1.56^a	2.46 ± 0.17^a
480	69.2 ± 4.6^a	29.1 ± 3.22^b	3.35 ± 0.63^c	32.4 ± 1.92^a	2.39 ± 0.11^a

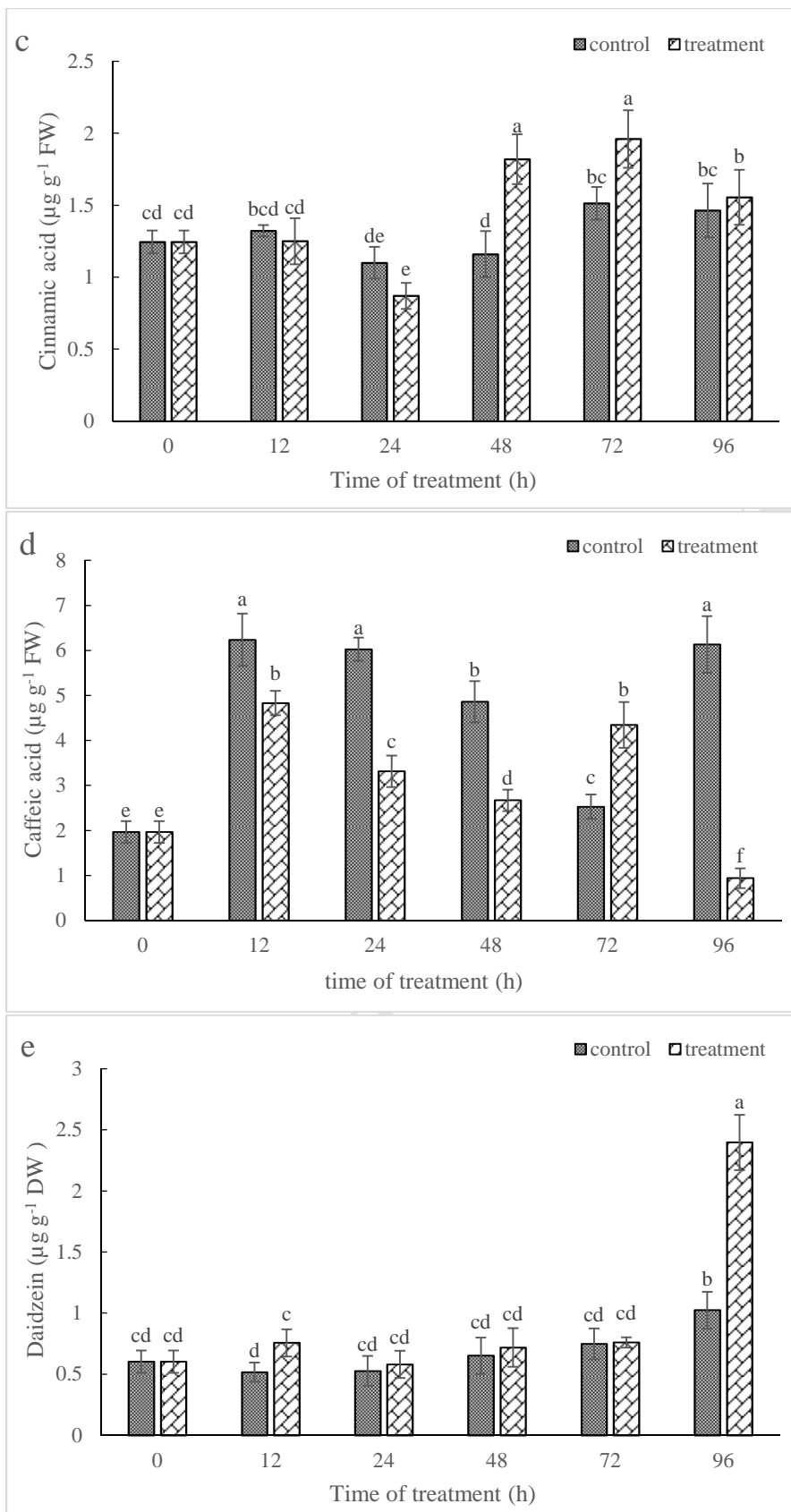
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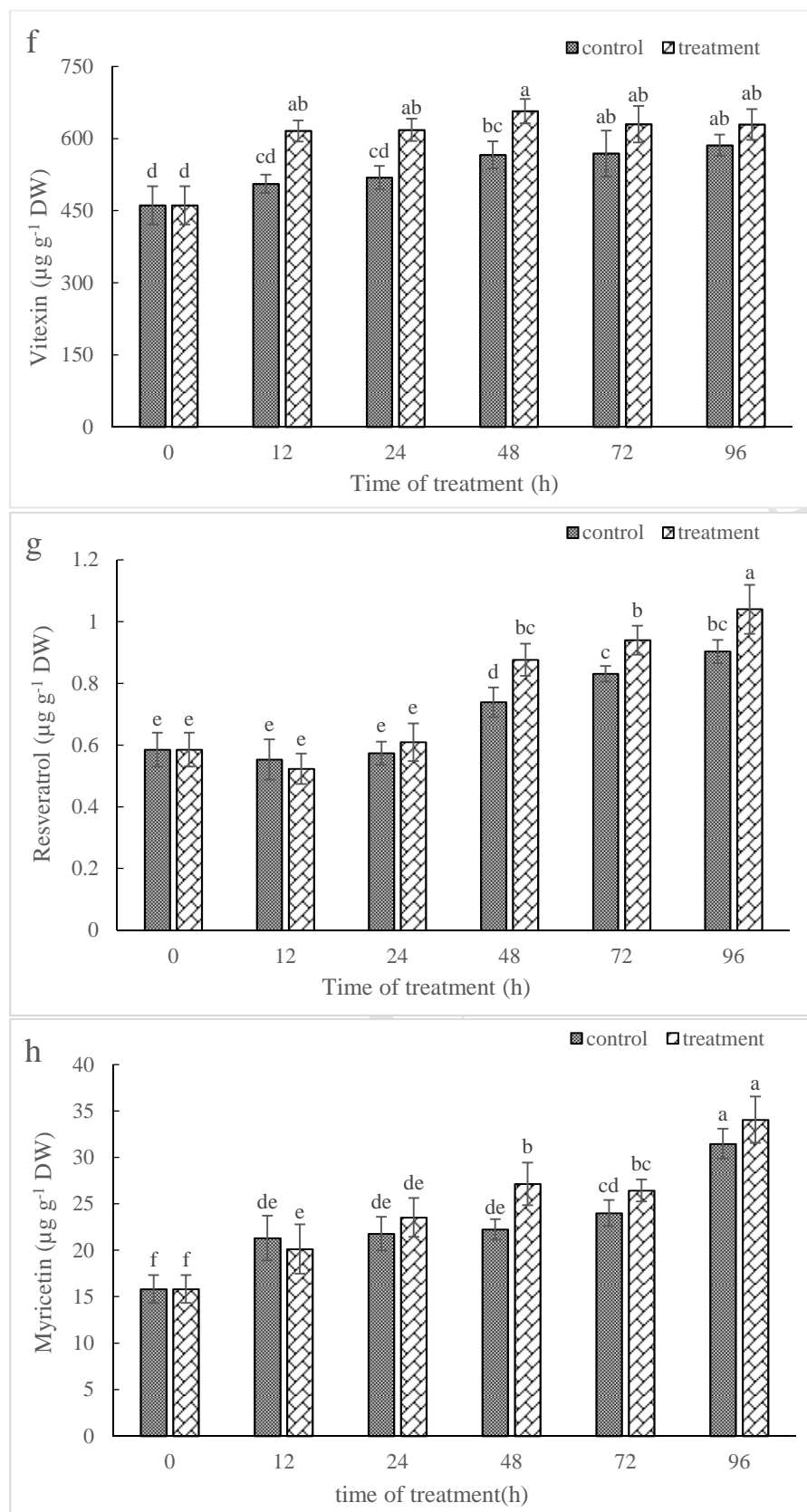
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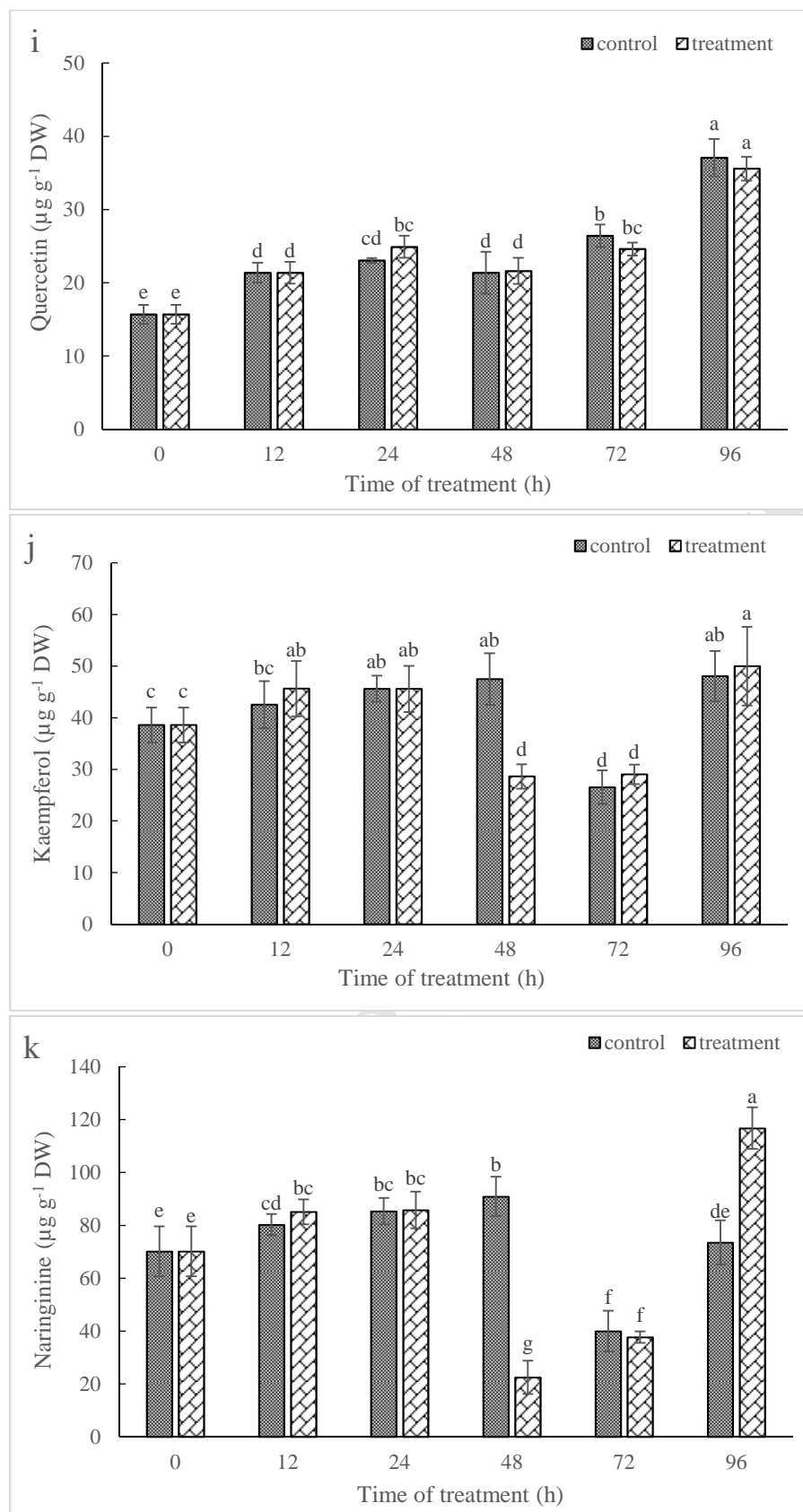
sample	Time-course [h]	NO content [nmol $\text{g}^{-1}(\text{DW})$]	H_2O_2 content [nmol $\text{g}^{-1}(\text{FW})$]	PAL activity [μmol CA mg^{-1} (protein) h^{-1}]	Total phenolics [mg g^{-1} (DW)]	Total amino acids [mmol $\text{g}^{-1}(\text{FW})$]
control	0	65.5 ± 4.10^b	25.32 ± 1.43^e	5.80 ± 0.43^f	18.33 ± 1.86^d	1.96 ± 0.13^e
	12	62.1 ± 4.82^b	27.14 ± 1.27^{de}	6.96 ± 0.68^{cd}	20.58 ± 1.27^{cd}	1.95 ± 0.11^e
	24	68.7 ± 5.63^b	27.19 ± 2.12^{de}	7.02 ± 0.32^c	21.55 ± 2.12^{cd}	2.14 ± 0.14^{de}
	48	68.2 ± 4.65^b	24.05 ± 1.36^e	7.58 ± 0.65^c	21.85 ± 1.58^{cd}	2.43 ± 0.10^{de}
	72	64.2 ± 5.24^b	29.19 ± 1.91^{cd}	6.56 ± 0.61^{de}	22.05 ± 0.90^{cd}	2.79 ± 0.22^d
	96	61.6 ± 4.33^{bc}	30.20 ± 0.94^{cd}	6.50 ± 0.48^{de}	24.75 ± 2.01^{bc}	3.11 ± 0.08^c
treatment	12	79.6 ± 4.23^a	43.93 ± 2.05^a	7.43 ± 0.30^e	21.38 ± 1.63^c	2.25 ± 0.09^{de}
	24	71.8 ± 4.91^b	38.51 ± 2.71^b	11.46 ± 0.49^a	26.32 ± 1.93^b	2.87 ± 0.17^c
	48	66.1 ± 3.96^b	31.92 ± 1.56^c	10.24 ± 0.77^b	27.39 ± 2.42^b	3.71 ± 0.26^b
	72	58.4 ± 6.21^{cd}	36.44 ± 3.09^b	6.26 ± 0.56^e	36.71 ± 3.34^a	4.32 ± 0.14^a
	96	53.9 ± 5.16^d	37.82 ± 2.48^b	5.47 ± 0.33^f	39.19 ± 2.71^a	3.65 ± 0.19^b

Table 2. The content of NO, H_2O_2 , Total phenolics, Total amino acids and PAL activity of *Prosopis* shoots under control and Pb treated conditions. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

Figures







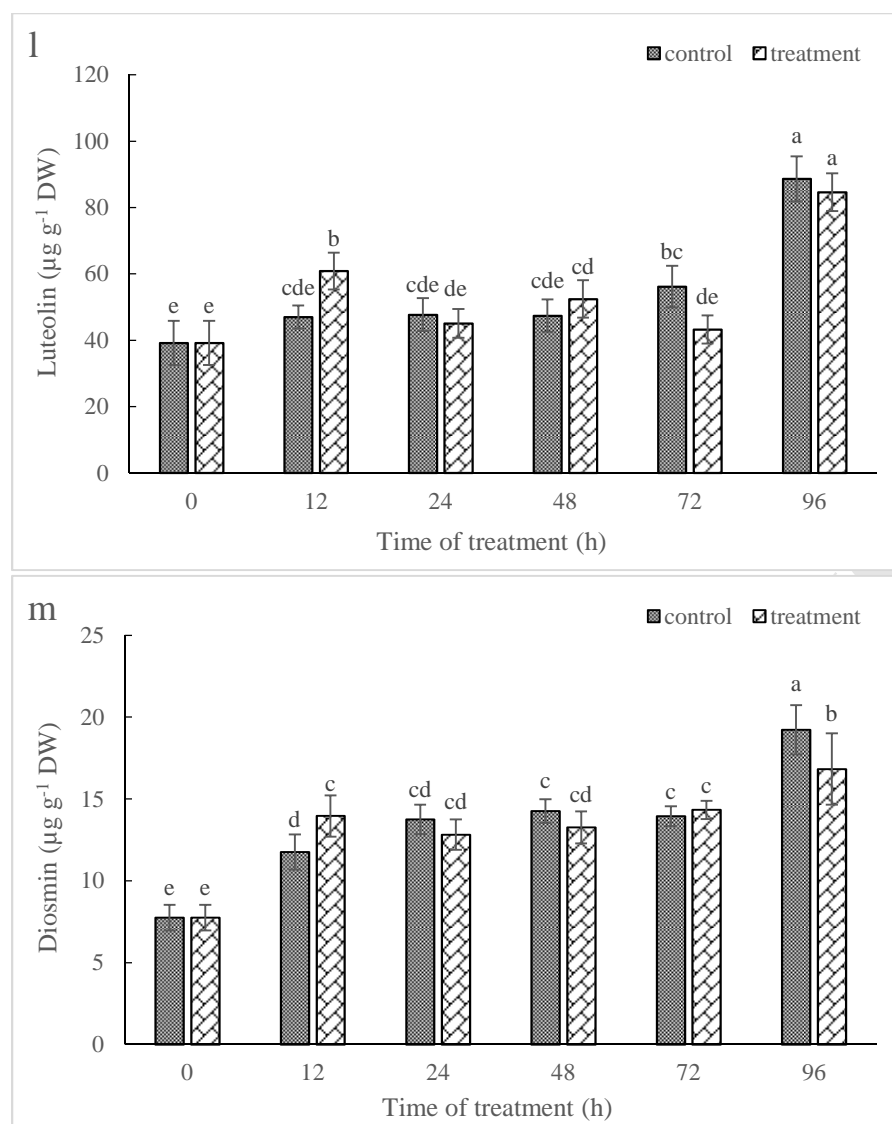
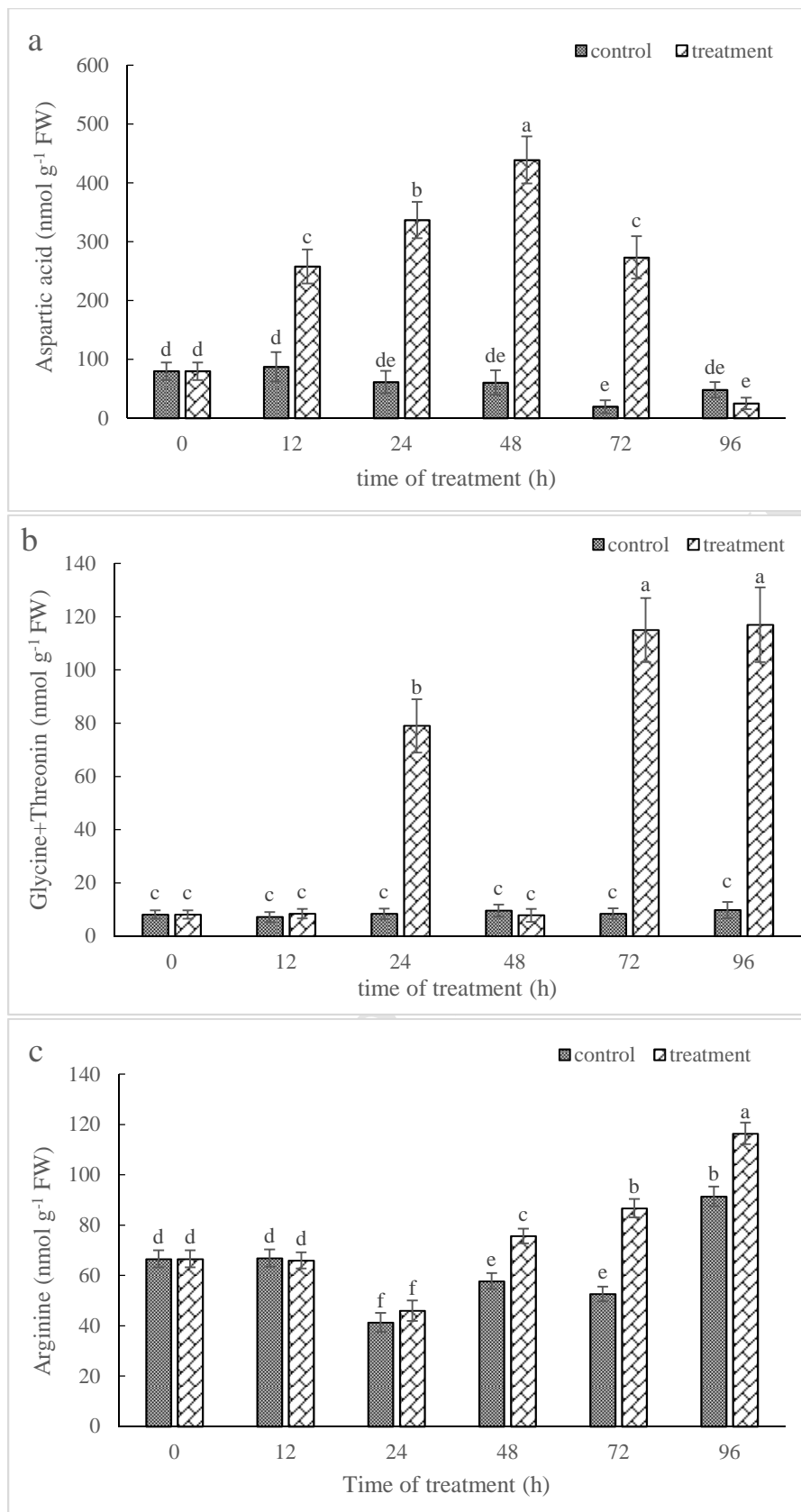
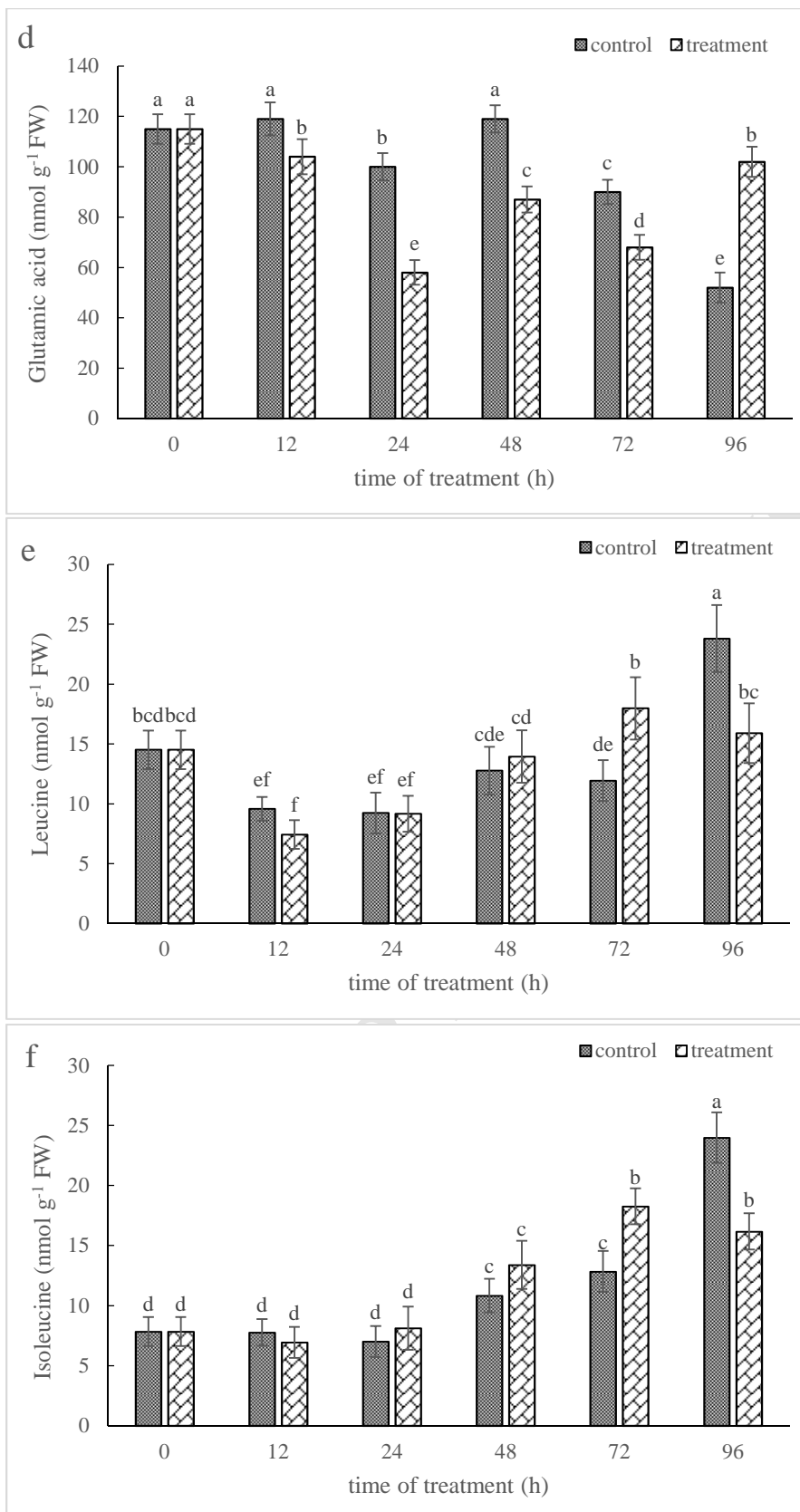
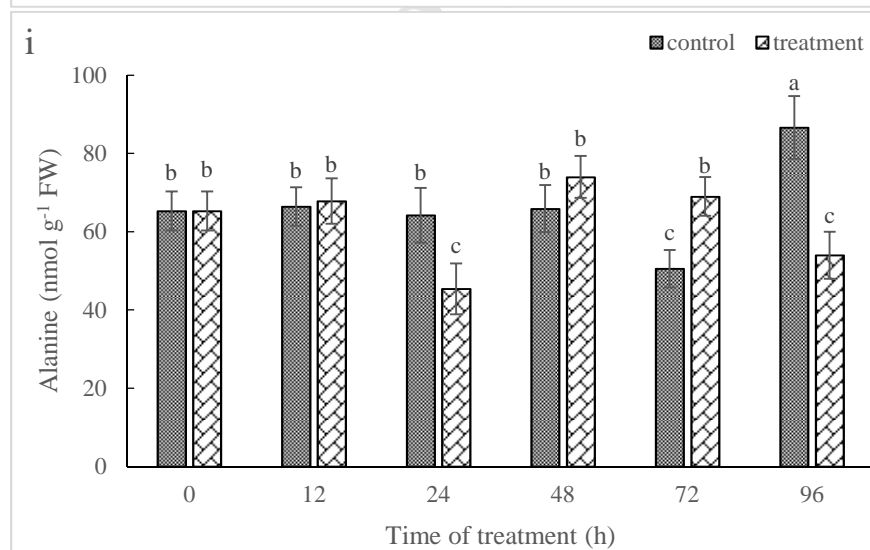
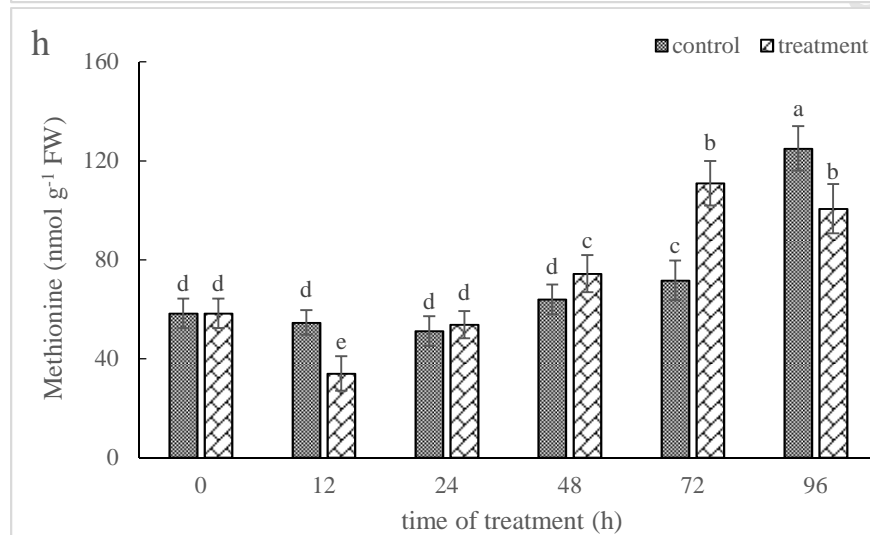
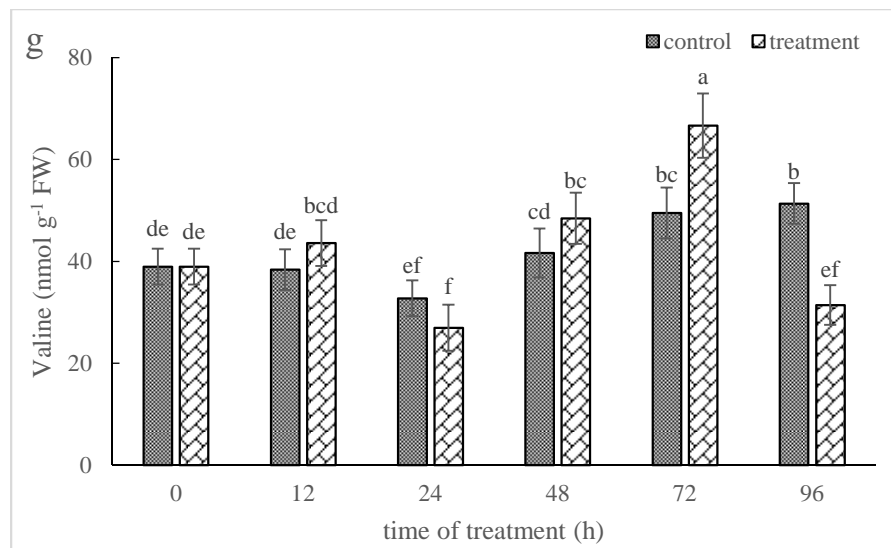
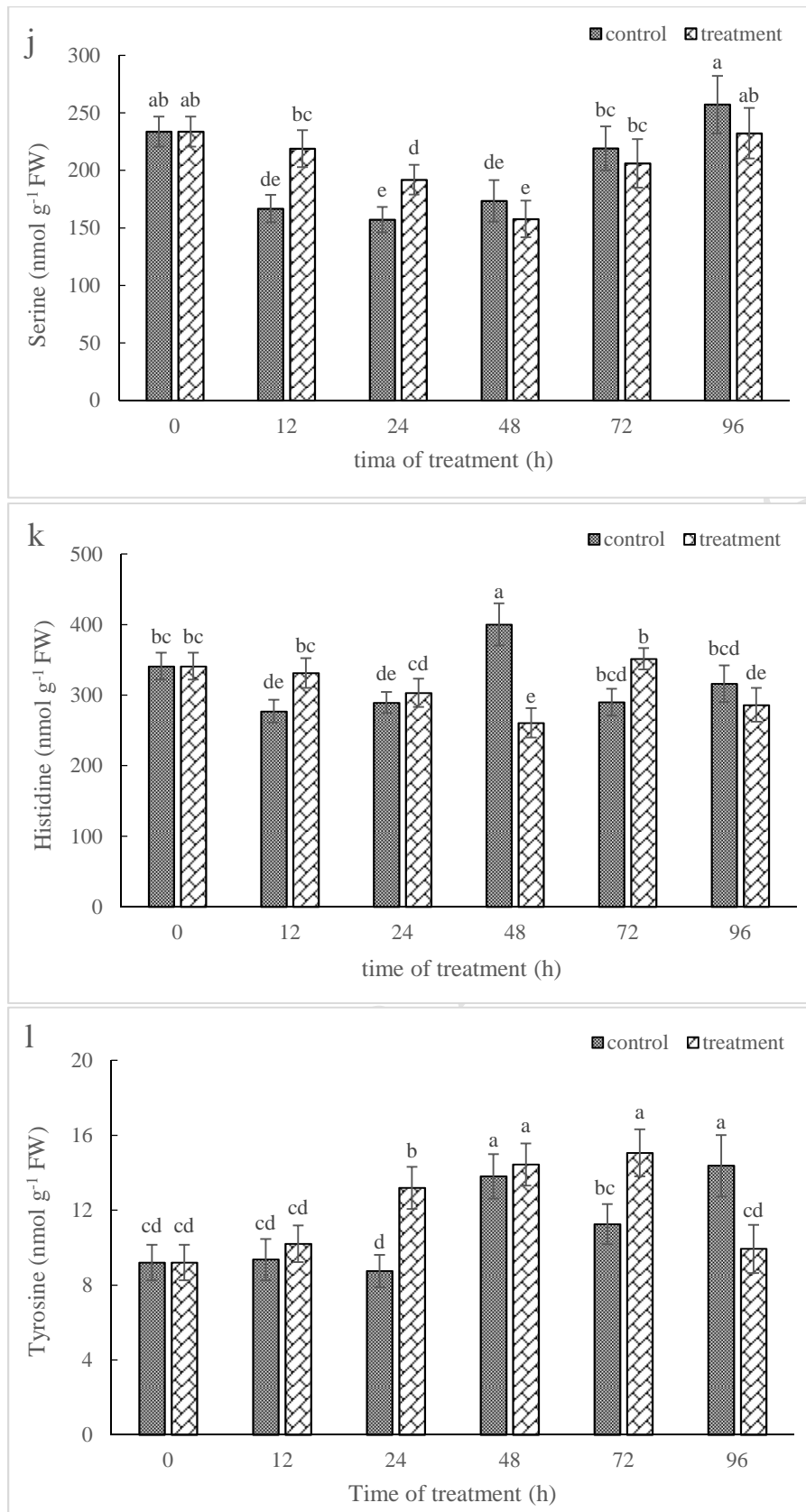


Fig. 1. Content of phenolic acids and flavonoids in *Prosopis* shoots: (a) salicylic acid, (b) ferulic acid, (c) cinnamic acid, (d) caffeic acid, (e) daidzein, (f) vitexin, (g) resveratrol, (h) myricetin, (i) quercetin, (j) kaempferol, (k) naringin, (l) luteolin, (m) diosmin. The plants were exposed to Pb treatment for 96 h. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.









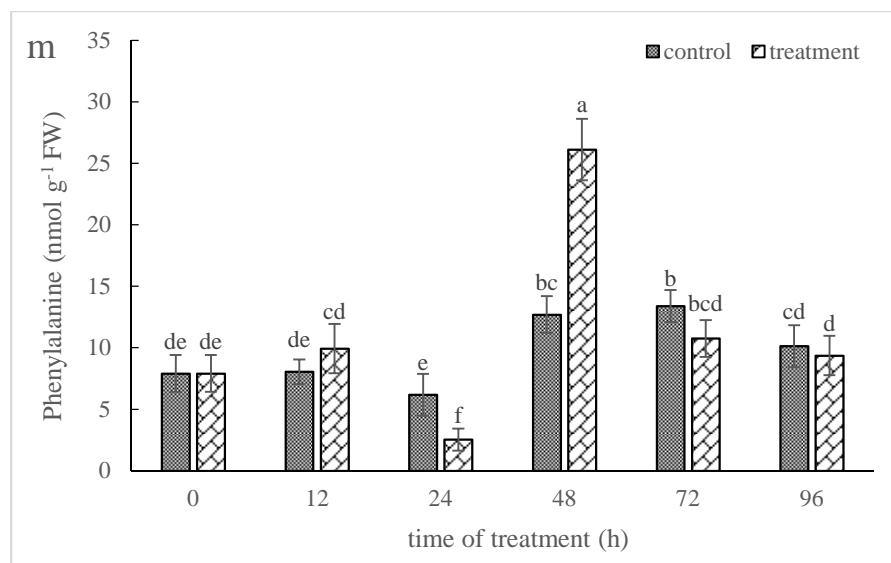


Fig. 2. Accumulation of free amino acids (nmol g FW, $n = 3$) in *Prosopis* shoots: (a) aspartic acid, (b) glycine+threonine, (c) arginine, (d) glutamic acid, (e) leucine, (f) isoleucine, (g) valine, (h) methionine, (i) alanine, (j) serine, (k) histidine, (l) tyrosine, (m) phenylalanine. The plants were exposed to Pb treatment for 96 h. Data are means \pm SD. Values within rows followed by the same letter(s) are not significantly different at ($P \leq 0.05$) level.

Highlights

- Endogenous signaling molecules contents (NO and H₂O₂) increased in *Prosopis farcta* shoots at the early times after lead (Pb) feeding.
- Phenylalanine ammonia lyase (PAL) activity enhanced at the early days of exposure to Pb.
- Following increased PAL activity, significant increases in phenolic acids and flavonoids; daidzein, vitexin, ferulic acid and salicylic acid were observed in the fed *Prosopis* with Pb.
- Pb treatment altered the composition and concentration of some free amino acids in *Prosopis* shoots.

Author contribution:

This research paper was accomplished with the collaboration of all authors. Somaieh zafari performed the experiments, analyzed data and wrote the manuscript. Mohsen Sharifi designed the study and supervised the study. Najme Ahmadian was the study advisor and established HPLC methods. Luis A. J. Mur provided consultation and wrote the manuscript.